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Review

Genetically-manipulated adult stem cells as therapeutic agents and gene delivery vehicle for wound repair and regeneration

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ABSTRACT

Wound therapy remains a clinical challenge and much effort has been focused on the development of novel therapeutic approaches for wound management. New knowledge about the way in which signals control wound cellular and molecular behavior has promoted the topical application of multipotent stem cells and bioactive molecules to injured tissue, for skin regeneration with less scar formation. However, limited clinical success indicates that the effective delivery of polypeptides and therapeutic cells, with controlled releasing profile, is a major challenge which is yet to be overcome. Recently, a technique in which the genetically-manipulated stem cells were used both as the therapeutic agents and the vehicle for gene delivery for wound treatment – a method which serves to provide regenerative cells and bioactive genes within an optimal environment of regulatory molecular expression for wound sites – has emerged as a promising strategy for wound regenerative therapy.
In this article, the roles of adult stem cells – as the therapeutics and the vehicles in these advanced biomimetic drug delivery systems for wound regeneration medicine – are scrutinized to indicate their mechanisms, characteristics, broad applicability and future lines of investigation.

Contents

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1. Introduction

Skin plays an extremely important role in providing an immune network and physical barrier against mechanical, chemical, and microbial factors from the external environment, as well as acting as a unique defense system against UV radiation (UV-R) (ii) through its pigments, secreted by melanocytes located in the bottom layer of epidermis. The effective healing of deep dermal wounds – including those which are chronic in nature or slow to heal – and the avoidance of scarring complications are challenging objectives which illustrate a general lack of efficient treatments for large wounds [1]. Emerging knowledge of the cellular activities involved in the wound healing process has advocated the topical delivery of large number of therapeutic cells, e.g. keratinocytes and fibroblasts, after propagation *ex vivo* for (i) the immediate coverage of the wound site, thereby stimulating the host to produce a variety of cytokines, (ii) the provision of the basement membrane, (iii) the prevention of dehydration and (iv) the activation of healing responses [2]. However, major problems have occurred, over the past years, with the topical delivery of cells or/and growth factors using traditional techniques, such as: (i) the lack of long-term integration of the cellular sheets, (ii) the incomplete healing and frequent generation of scar tissue, (iii) the inadequate appendage contribution and (iv) overt rejection.

Meanwhile, reduction in tissue growth factors and cytokines has been shown to significantly delay wound healing and to initiate scar formation [3], which stimulated the widespread application of growth factors in wound therapy. However, the clinical effectiveness of traditional topical administration of growth factor peptides remained discouraging, suffering from the inherent loss of drug activity, due to the combined effects of physical inhibition and biological degradation of the bio-molecules.

Recently, gene transfer technology – in which genetically-modified cells synthesize and deliver the encoding growth factors to the wound site in a time-regulated and locally-restricted manner – has been experimentally demonstrated to be a valuable means of overcoming the limitations associated with traditional, topical delivery of recombinant proteins [4].

More interestingly, in recent years, the use of genetic recombinant stem cells and biomimetic nanostructured scaffolds for the development of novel biomimetic drug delivery systems, has received widespread attention as a promising strategy for wound treatment, in which multipotent stem cells, encoded with plasmid DNA of polypeptides, are used both as the cellular therapeutic medium and the vehicle for the delivery of functional genes to the wound site. In this article, the roles of bioactive molecules and tissue-specific adult stem cells involved in wound healing are reviewed, followed by a discussion of their application in the construction of biomimetic drug delivery systems for skin repair and regeneration.

2. Functions of bioactive molecules in wound healing

2.1. Bioactive molecules in wound healing

Normal wound repair of tissues and organs involves four overlapping yet distinct stages. These are: (i) the initiation of hemostasis and inflammatory response; (ii) the formation of granulation tissue, epithelialization and angiogenesis, as well as the synthesis of extracellular matrix proteins; (iii) the maturation and remodeling of connective tissue and (iv) the recovery of the wound strength, accompanied with fibrosis (see Fig. 1 as an overview of the wound healing process). It can be seen that wounds include a complex signaling network, which involves numerous molecules. The cellular and molecular mechanisms which critically influence the wound repair quality, and how their related responses can be manipulated by therapeutic intervention, represent key questions in wound genetic- or cellular therapy. Currently, many experimental studies have helped to clarify the influences, on wound repair and healing quality, of growth factors, cytokines, chemokines, transcriptional factors and newly-identified bioactive molecules [5–7]. (Fig. 1 showed a list of the participation and up-regulation in most conditions of the bioactive molecular effectors in the wound healing stages of inflammation and hemostasis, re-epithilialization, angiogenesis and remodeling.) However, a critical issue in the application of these functional molecules in wound healing has been the development of a strategy to optimize the delivery of these polypeptides, in order to maximize their therapeutic efficacy. In recent years the molecular genetic approach – using genetically-manipulated cells as the vehicle for delivery of the desired growth factor – has been experimentally demonstrated to be a useful strategy. Also, in contrast to employing many differentiated cell phenotypes, stem cells are potentially permanent residents of the wound site and will help to sustain the expression of the transfected genes and bioactive proteins. This knowledge may create new opportunities for the clinical application of versatile molecular regulators in tissue healing.

3. Adult stem cells involved in wound repair and regeneration

Besides emerging knowledge about the participation of various molecular effectors, the identification of the roles that stem cells play in wound regeneration is probably the most active field in wound healing mechanistic investigation. Currently, stem cells derived from epidermis, dermis, hair follicle, adipose tissue and bone marrow have been demonstrated to contribute significantly to the maintenance of skin renewal, homeostasis and regeneration. Their functions in wound healing include: (i) acting as multiple players in re-epithelialization; (ii) being progenitor cells with differentiation potential from mesenchymal to epithelial phenotypes; (iii) being endothelial cell precursors for revascularization; (iv) expressing the fibroblast-specific collagen genes that contribute to wound remodeling; (v) promoting repigmentation; and (vi) causing hair follicle neogenesis [8,9]. The characteristics and roles of the cutaneous stem cells that are closely involved in wound healing are reviewed as follows.

3.1. Epidermis and hair follicle-derived stem cells

The maintenance of homeostasis in the epidermis is possible via the self-renewing ability of the epidermal stem cell (ESC) population, which gives rise to differentiated keratinocytes. It is believed that ESCs play an important role in cellular regeneration, wound healing, and the pathogenesis of skin cancers. ESCs reside in the basal layer of the epidermis and are known to be responsible for regenerating the epidermal tissue during an individual's lifetime because of their great proliferative capacity. Under physiological circumstances, ESCs serve as a cell pool for the cyclic regeneration of the anagen hair bulb and they also regenerate the sebaceous gland and the epidermis after injury [10,11]. The bulge region of the hair follicle is firmly believed to be a niche of multipotent stem cells. Subsets of these follicle-derived multipotent stem cells mainly include the hair-follicle-derived epithelial stem cells, mesenchymal stem cells, melanocyte and nestin-positive stem cells. These stem cells can be activated and they then migrate out of the hair follicles to the site of a wound, to replenish lost cells and repair the damaged epithelium, the follicle and the sebaceous gland [12]. Hair-follicle stem cells (HFSCs) are neither necessary for epidermal survival, nor does their absence prevent re-epithelialization, but they seem to enhance the early stages of wound closure [13]. Additionally, there is increasing evidence that HFSCs are a source of epidermal and dermal cell populations. However, it is surprising to find that the majority of HFSCs do not persist in the regenerated epidermis and this may suggest that HFSCs and ESCs are intrinsically different, in that the ESCs seem better suited to establishing long-term epidermal units [14]. Because of the marked ability

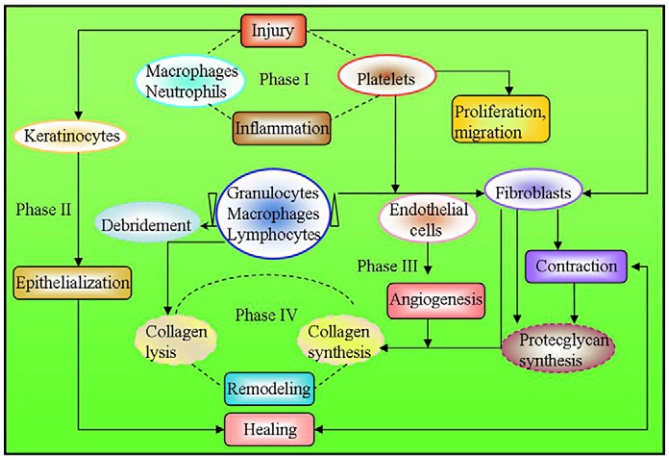

Fig. 1. Overview of the adult wound healing process: Phase I, hemostasis and inflammation with many immune cells, cytokines, chemokines and growth factors involved; Phase II, primary repair with proliferation, migration of cells and re-epithelialization by keratinocytes; Phase III, angiogenesis mainly regulated by VEGF family; Phase IV, remodeling with various miRNAs, growth factors and connexin attend.

of ESCs and HFSCs to reconstitute the epidermis and hair follicle, they have been viewed as being the ultimate source of most cutaneous epithelial lineages and as having the potential to generate more hematopoietic cell types and to repair the epidermis.

3.2. Bone marrow-derived stem cells

Normal skin is the target for bone-marrow-derived cells (BMSCs) both from the hematopoietic and mesenchymal stem-cell pools. The ability of BMSCs to contribute to the regeneration of different tissues has been demonstrated both *in vitro* and *in vivo* [15]. After a tissue injury, hematopoietic and mesenchymal stem cells are mobilized from the bone marrow into the pool of circulating cells. These cells then migrate to the injury site, where they regulate the proliferation and migration of epithelial cells and dermal mesenchymal cells during the early inflammatory phase [16]. Bone marrow also provides self-renewing stem cells that differentiate into fibroblast-shaped cells and the dermal keratinocytes phenotype; they also increase the number of regenerating appendage-like structures, which serve as the precursors of permanent cutaneous appendages [17]. Governed by the Wnt/b-catenin pathway, the accumulated BMSCs are able to differentiate into fibroblasts [18] and they are stably represented in dermal wounds throughout the repair process – especially during tissue remodeling – greatly contributing to the laying-down of collagen in the ECM [19]. Bone-marrow-derived hematopoietic stem cells have long been recognized as a precursor to blood cell lineages [20]. However, they have recently been identified as giving rise to versatile cell phenotypes, e.g. fibrocytes [21] and smooth muscle cells [22].

3.3. Adipose derived-stem cells

Adipose derived stem cells (ADSCs) are an abundant, readily available population of progenitor cells that reside in adipose tissue [23]. ADSCs are multipotent stem cells with the capacity to facilitate angiogenesis in deeper wounds. The increase in tissue vascularization – by differentiation into endothelial cells – is thought to be their most important effect on wound healing [24]. They have also been observed *in vitro* [25] to secrete significant quantities of angiogenic and anti-apoptotic factors, including the vascular endothelial growth factor (VEGF) and the hepatocyte growth factor (HGF). By promoting the proliferation and migration of dermal fibroblast proliferation and by enhancing the collagen synthesis and re-epithelialization, ADSCs and their secretory factors have shown great promise for their use in skin repair and regeneration [26]. Because ADSCs have many properties in common with bone-marrow-derived mesenchymal stem cells, ADSCs may ultimately represent a valuable therapeutic option in tissue rescue and repair, in preference to the BMSCs: this is because of their wide availability, their pro-angiogenesis and their anti-apoptotic factor secretion effects, their immunomodulatory effects, and their capacity for multi-lineage differentiation and ready expansion [27].

4. Adult stem cells as therapeutic agents for wound treatment

A wide range of evidence from animal studies indicates that engrafting within multiple tissues in the body gives rise both to tissue-specific cells and releasing soluble factors that trigger the tissue's own endogenous repair pathways. It is now well known that stem cells can significantly stimulate the regeneration of endogenous cells and promote tissue recovery, which has undoubtedly shown cutaneous stem cells to be superior as powerful healing agents for wound regeneration. Fig. 2 shows the differentiation characteristics of several tissue-specific adult stem cells for wound healing, because of their potential, demonstrated by several studies [28], to generate “skins” containing components observed in natural tissues, such as sweat gland and hair follicle. Hitherto, the implantation either of autologous or allogenic bone marrow-derived stem cells (BMSCs) has been demonstrated to accelerate wound healing and increase the formation of new vessels and granulation tissues [29,30]. The main disadvantage of BMSCs, however, lies in the obvious impairment of their differentiation abilities with increasing age [31]. In contrast, the easy isolation, relative abundance and multipotency of adipose-derived stem cells (ADSCs) that are independent of the serum source, provide them with a significant potential to rescue tissue from damage [32]. ADSCs not only possess the capacity to differentiate into skin lineages under appropriate conditions but also to release the angiogenic factors which stimulate angiogenesis [33]. Epidermis derived stem cells (ESCs) are largely quiescent in the skin, but they can be activated to regenerate large numbers of skin cells and they have shown the potential for providing a superior source for skin tissue engineering [34]. Purified ESCs have been shown to have a dramatically-enhanced capacity to regenerate functional human epidermis [35]. Hair follicle-derived stem cells (HFSCs) have been applied in skin equivalents because the regenerative capacity of these cells has been shown to induce hair follicle formation within tissue grafts [36]. Recently, it has been suggested that hematopoietic stem cells are a source for skin engineering, in that they promote a perfect repair after deep and extensive burns and that they make a significant contribution to angiogenesis [37]. As a large, untapped stem cell resource, the use of bone marrow-derived hematopoietic stem cells for burns and other wounds may open up the possibility of providing cheap and accessible care for burns and wounds for an increasing number of patients. Other advantages of hematopoietic stem cells include their ethical acceptability, native immune status and relatively unshortened telomere length [38]. Recent studies have shown that adult stem cells have the ability to enhance fibrous matrix degradation by the induction of matrixmetallo proteinases, which also suggested that adult stem cells may be effective therapeutic tools for the treatment of fibrosis complications [39]. It is also worth mentioning that, although adult stem cells represent an attractive alternative because they lack the above-mentioned problems and are feasible for autologous transplantation, they appear to possess much lesser pluripotency than was suggested earlier [40,41]. Meanwhile, one obvious problem in the large-scale *in vitro* production of terminally specified cells from differentiating stem cells is their low final yield. Attempts to enhance this by external introduction of growth- and/or signaling factors essential for differentiation of the cells of interest have therefore been investigated to solve these issues.

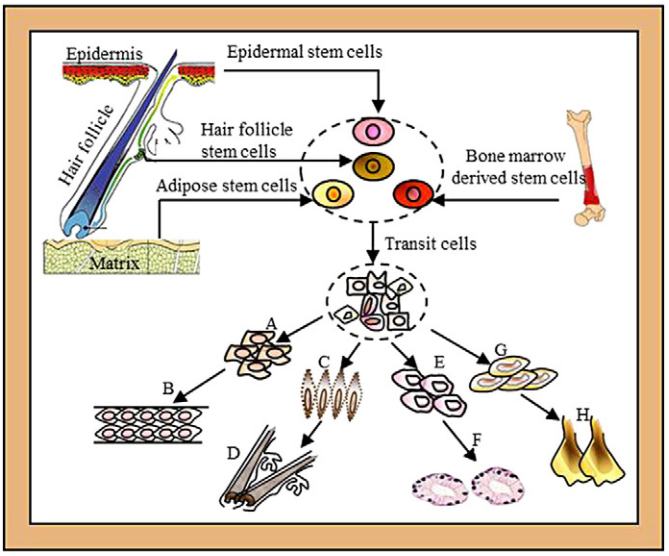

Fig. 2. Differentiation potential of cutaneous stem cells in skin regeneration (A. Keratinocytes; B. Epidermis; C. Keratinocyte containing melanin; D. Hair follicle; E. Sweat gland cells; F. Sweat gland; G. Sebocytes; H. Sebaceous gland).

5. Adult stem cells as a vehicle for gene delivery in wound therapy

5.1. Rationale of the application of stem cells as a vehicle for gene delivery to wound sites

The presence of high and sustained levels of signaling factors has been demonstrated to be one of the most important factors for *in vivo* healing because of the motility, differentiation, organization, proliferation and apoptosis of the cells associated with tissue formation. This is because they are greatly influenced by cytokines and ECM proteins in the local micro-environment. The topical application of exogenous active polypeptides, populating the wound environment with ample levels of signaling factors is, therefore, a widely-studied strategy for wound treatment. However, because most polypeptides easily lose their bioactivity because of physical inhibition and biological degradation, clinical trials had little success with their direct, topical application. With the development of gene delivery technologies, direct transfer of plasmid-encoded target genes with viral or non-viral vectors to the *in vivo* wound sites – or use of *ex vivo* living cells, in which genetically-modified cells synthesize the active peptides or express the genes of interest after transplantation – has been investigated as an alternative strategy for delivery of the growth factors [42]. Viral vectors have been used as gene delivery vehicles due to the specificity with which they can bind to and infect cells [43] but viral infection-associated toxicity, immunologic compromise and possible mutagenic or carcinogenic effects make this approach potentially dangerous [44]. Non-viral delivery systems are easy, simple, direct, inexpensive and do not require *ex-vivo* manipulation. However, problems of transient gene expression, inability to selectively deliver genes to specific cells and the variability in the level of gene expression remain unsolved problems in the use of non-viral vectors [45]. In recent years, the use of cells as a gene delivery vehicle was demonstrated to be advantageous in precise and flexible manipulation, with no more inflammation- and infection risk than would be obtained by *in vivo* means [46]. In this approach, either the plasmid DNA or the genes encoding the certain signaling factors (growth factors or cytokines) are introduced into the cells that act as the gene delivery vehicles. The required functional mediators can be expressed and secreted spatially and temporally by the transfected cells [47]. In this strategy, the problem of transfection efficiency may not be a serious issue, as the cells that do take up the gene are selectively cultured and those that do not can be eliminated. The most common technique for addressing mediated gene delivery is to use allogeneic cells, which requires more specialization and a more complex treatment procedure. From a financial standpoint, many procedures involve high cost, as well as requiring appropriate facilities and technologies, and the strategy thus lacks support as suitable for large-scale application. Until recently, the percentage of patients undergoing cell-meditated gene therapy in clinical trials was still rather low. In recent years, transplantation of homologous cells for tissue repair and regeneration has been investigated to circumvent the use of allogeneic cells. Increasing numbers of studies [48,49] have demonstrated the possibility of isolating and culturing allo-cells and utilizing them as the gene delivery vehicle, despite the presence of an immune reaction, which may achieve immunotolerance in different types of transplantation.

Recently, adult stem cells are thought to be an ideal vehicle for delivering genes to the injured sites. Stem cells can be defined to be embryonic or adult in nature. Embryonic stem cells are derived from mammalian embryos in the blastocyst stage and have the ability to generate any terminally-differentiated cell in the body [50]. However, in spite of their tremendous potential, there are ethical concerns and issues over their use. By comparison, adult stem cells (ASCs) can be harvested from patients themselves and the ethical barrier for clinical application is low: more and more clinical experiments on cell-based tissue regeneration have been conducted with adult stem cells. Currently, various adult stem cells have been investigated for their potential as vehicles for gene delivery to the target tissue or organs because of a number of characteristics: (i) cutaneous stem cells derived from skin, adipose tissue and bone marrow have high migration potential and appear in increasing numbers in the wound site [51]; (ii) their unlimited multiplication properties, with stable phenotype and monolayer characteristic *in vitro*; (iii) their site-specific differentiation capacity into skin linkages which have long survivability [52]; (iv) ready availability with less invasive methods and *in vitro* multiplication properties [53]; (v) immunosuppressive effects and low a mutation rate; (vi) lower neurotoxicity or tumorigenicity; (vii) immunomodulatory/anti-inflammatory properties [54] and (viii) mesenchymal stem cells (MSCs), engineered to express higher levels of proteins known to be beneficial for the tissue in question, have produced even better results than unmodified MSCs [55].

5.2. Genetic manipulation of adult stem cells

In most conditions, the stem cell-based gene delivery strategy involves three main steps. First, the stem cells are isolated and cultured *in vitro*. Second, the therapeutic gene is transduced into the stem cells by applying methods similar to those used in a direct gene transfer. Finally, the genetically-modified stem cells are returned to the patient. For these cells to act as an efficient gene delivery vehicle, efficient gene transfer to the stem cells is prerequisite. Various viral or non-viral vectors, based on a two-dimensional system, have been widely investigated for gene transduction to the stem cells. Interestingly, a novel three-dimensional scaffold-based transfection system has been recently reported and this may well be a promising strategy for stem cell recombination.

5.2.1. Viral vectors

Various viral vectors are currently, being used for the transfection of cutaneous stem cells (e.g. retroviruses, adenoviruses, and adeno-associated viruses) and they efficiently produce a wide range of cytoplasmic, membrane-bound and secreted protein products. The strategy with viral vectors is to generate a replication-defective particle by replacing some or all of the viral genes with the gene of interest. The choice of viral type depends, to some extent, on whether the permanent- or the transient expression of the gene product is desirable [56]. Recombinant retroviruses are the most commonly used vehicle for

gene transfer to the wound site. Retroviruses are capable of simultaneously transferring genes to large numbers of cells at high efficiencies. Their other advantage is that they allow persistent gene expression. However, one major disadvantage of retroviral-mediated gene transfer is that only a gene of limited size (<6 kbp) can be packaged and transferred [57]. Adenoviral vectors have been demonstrated to have the highest gene transfer efficiency *in vivo*. The major limitation of adenoviral vectors may be the cytotoxicity of viral proteins and the host cellular immune response to the adenoviral proteins, causing local inflammation and destruction of the transduced cells [58]. Adeno-associated viruses (AAV) can transduce a wide range of dividing and non-dividing cells with the wild-type virus integrating into a specific position on the chromosome. The disadvantages of AAV vectors are that: (i) they package only small transgenes; (ii) the preparation of AAV is frequently contaminated with wild-type adenovirus; (iii) immune responses can occur with AAV administration and (iv) many individuals have prior immunity [59]. Viruses are the vectors which provide high transfection efficiency and a rapid transcription of the foreign material inserted into the viral genome. But many clinical trials in which viral vectors have been used have been interrupted because the application of these vectors induced various unexpected adverse effects, including mutagenesis, carcinogenesis and immune response. Non-viral transfection strategies, e.g. cationic liposome and nanoparticle-based vectors, were therefore extensively explored and they offered an alternative choice for gene transfer. It is worth mentioning that non-viral transfection is particularly advantageous for wound gene therapy because it expresses the therapeutic protein in a safe and relatively transient way.

5.2.2. Non-viral vectors

Cationic liposomes and polymers are the non-viral vectors which are at present receiving the most attention as reliable and relatively efficient vehicles for gene transfer [60]. Nano-sized lipoplexes or polyplexes are formed by combining cationic liposomes or polymers with nucleic acids. The particles bind to the cell surface by non-specific, electrostatic interactions between the positively-charged complexes and the negatively-charged cell surface and they enter cells by endocytosis or endocytosis-like mechanisms. Once inside, the pH of the endosome compartments decreases from 7 to 5.5 and a proportion of the bound nucleic acids escape from the early endosomes into the cytosol. The lipoplexes or polyplexes then dissociate and the released plasmid DNA enters the nucleus by either of two hypothesized pathways: (i) passive DNA entry into the nucleus during cell division when the nuclear membrane disintegrates temporarily or (ii) active transport of the DNA through the nuclear pores [61].

5.2.2.1. Cationic liposome-based vectors. Liposomes are like vesicles that can be used to encapsulate anything from drugs to plasmids. Liposomes form lipoplexes when they interact with DNA. Because of their opposite surface charge, cationic liposomes can form an overall positively-charged complex with negatively-charged DNA. The resulting positively-charged biocompatible lipid-DNA complexes (lipoplexes) do not face any electrostatic barrier in penetrating the negatively-charged biological cell surfaces and they are endocytosed by the cell plasma membrane. In addition, cationic liposomes also protect DNA from attack by the enroute deoxyribonucleases. Lipid-based vectors are commercially available as transfection reagents (e.g. Lipofectamine 2000). However, despite the great progress achieved in lipid-based gene delivery, which has eliminated the serious immunogenic concern in gene therapy, cellular barriers are still a major impediment and the low frequency of stable transfection is still the major drawback of liposome vectors [62]. Extensive efforts have been made to enhance their efficiency, such as the synthesis of new cationic lipids and the development of a new approach to the formulation of complexes.

5.2.2.2. Cationic polymer-based vectors. Cationic polymer-based vectors are widely used as transfection reagents because their cationic nature is advantageous for condensing with DNA. They are amphiphilic molecules, which contain a positively-charged polar head group, linked to a hydrophobic domain via a connector combining with DNA to form a particulate complex. Polyplex has the obvious advantage of compressing DNA molecules into a relatively small size and is capable of transferring genes into the targeted cells. This can be crucial for gene transfer because a small particle size may be favorable for improving transfection efficiency. Cationic polymers can protect DNA from enzymatic degradation, as well as facilitating the cell uptake and endolysosomal escape [63]. Various polymers, e.g. polyethylenimines (PEIs), polylysine and polyamidoamine dendrimers are currently being widely investigated in gene delivery applications. However, the cell cytotoxicity, slow degradability and the low transfer rate of targeted genes by cationic polymers will need to be corrected before their clinical application is viable [64].

5.2.2.3. Cationic polymer conjugations. Currently, many efforts are being made to improve the biocompatibility of cationic polymers by increasing their solubility, biodegradability and chemical homogeneity. For example, PEIs possess poor solubility of their complexes with DNA. PEIs with higher molecular weights are easy to precipitate and are cytotoxic to cells but those with higher N/P ratio exhibit an attenuating effect on subsequent gene expression. In order to address these issues, we linked the PEI (25 kDa) with less toxic, biocompatible drug carriers, e.g. β-cyclodextrin (CyD), polyethylene glycol (PEG), or chitosan (CHI). The cytotoxicity of these conjugations has been shown to be significantly reduced to 0–60%, in comparison with 85%–100% for unmodified PEI. The transfection efficiency of these conjugations was enhanced 2.5- to 3.5-fold compared to unmodified PEI or 1000-fold compared to chitosan alone [65–67] to BMSCs. For another example, epidermal stem cells are difficult cells to transfect, and this feature may result from their slow cycle and, until now, little data has been available about their transfection with non-viral vectors. In our preliminary study, CYD-PEIs have induced a threefold-higher transfer rate of the reported genes and the enhanced endosomal fluorescence of FITC-DNA, in comparison with those of the Lipofectamine-2000 agent-treated group and this suggests the potential of CYD-PEI in the recombination of ESCs (data not shown). The underlying mechanism is currently under investigation.

5.2.2.4. Cationized polysaccharides. Cationized polysaccharides, e.g. spermine-dextran and spermine-pullulan are several of the novel non-viral vectors which we have constructed. It was shown that complexation with the spermine-dextran [68] or the spermine-pullulan [69] vector enabled plasmid DNA to decrease the apparent size of the vector–DNA complex, until it was small enough to internalize into cells by way of a sugar-recognizable receptor and to enhance the expression level of plasmid DNA. Currently, spermine-dextran and spermine-pullulan have been demonstrated to be promising non-viral vectors (with the added benefit of biodegradability) for enhancing the gene expression of mesenchymal stem cells and dendritic cells through receptor-mediated endocytosis. They have wide potential in tissue regeneration and cancer target therapy [70–72]. As with *in vivo* behavior, the major obstacles to the application of non-viral vectors may include their toxicity and relatively low transfection efficiency. Strategies to realize the targeting effects of non-viral vectors through topical delivery, the conjugation of the ligand and the control of the size of complex particles have been applied to formulate the modified non-viral delivery system. Currently, we have demonstrated that the transplantation of adrenomedullin-gene-engineered BMSCs by spermine-dextrane has apparently enhanced anti-apoptotic and angiogenic effects of BMSCs in a rat model of myocardial infarction [73]. In another preliminary study,

the spermine-pullulan-engineered BMSCs have exhibited promising target effects and anti-tumor effects in a rat model of lung carcinoma. The *in vitro* and *in vivo* efficiency of cationized polysaccharides engineered ESCs in skin regeneration is also under examination.

5.2.2.5. Optimization of two-dimensional transfection systems. Despite the development of various materials and novel non-viral vectors, research trials using non-viral gene carriers do not, until recently, achieve the expected results in terms of transfection efficiency. The level of gene expression is much lower than that of viral carriers and this is still a challenge to be overcome. It is the fact that vectors may be the most important element that influences the transfection efficiency. Many studies have, however, demonstrated that: (i) the cell culture condition with or without serum, (ii) the order of adding gene complexes and cells and (iii) the gene transfection time apparently influence the transfection efficiency. Because serum is critical for the *in vitro* culture of stem cells, the starvation of stem cells during the transfection process may exert an adverse effect on the growth of undifferentiated adult stem cells [74], which will, in turn, decrease transfection efficiency. It was reported that non-viral gene complexes enter the nucleus preferentially upon the disassembly of the nuclear envelope during mitotic cell division. This means that the presence of serum during transfection may facilitate the delivery of plasmid DNA to the nucleus by promoting the proliferation of MSCs. It was also recognized that non-viral carriers do not have any mechanism for nuclear internalization. Therefore, it is possible that, when the nuclear membrane disappears in cell division, the complex of non-viral carrier and plasmid DNA eventually enters the nucleus for gene transfection [75]. Based on this mechanism, the transfection efficiency of plasmid DNA by the non-viral carrier will increase with the proliferation of cells to be transfected. In the conventional procedure of gene transfection, the non-viral carrier is complexed with a plasmid deoxyribonucleic acid (DNA) and then added to the cell culture medium for transfection. In this case, serum cannot generally be added to the culture medium, although it is essential to maintain the correct biological conditions for cell culture. This is because the plasmid DNA carrier complex often interacts with the serum components, thereby leading to suppression of gene transfection [76,77]. Therefore, in order to develop a new transfection system with better cell culture conditions in the presence of serum, we coated the non-viral vector complex of a plasmid DNA on to a culture substrate together with a cell-adhesion substance; the cells were then cultured on the complex-coated substrate. The process is termed the reverse transfection system. In this method, cells are cultured in the presence of serum, which may enable the cells to proliferate more efficiently under better culture conditions and result in an enhanced gene transfection. Interestingly, it has been observed that there was a remarkable decrease of gene expression with the addition of serum by the conventional method. In comparison, the improvement of transfection efficiency in the presence of serum by using the reverse transfection method has been shown to be beneficial for the transfection of stem cells. Meanwhile, cell viability after the reverse transfection culture was significantly higher than after the conventional transfection culture. It was thought that the use of an anionic gelatin coating on the cell substrate could act as a shelter for the nanoparticles, to avoid the adsorption of proteins in the serum and thus the transfection process could be less affected with the addition of serum. Cells always make contact with the complex in reverse transfection culture, which is different from the contact time of 6 h in the conventional transfection culture, and this may be another advantageous condition for high transfection [78]. All these results are a reminder that, in addition to the synthesis of novel materials for vector constructions, it is important to optimize the transfection parameters and the procedures in order to maximize the efficiency of the two-dimensional system, and this deserves further mechanistic study.

5.2.3. Three-dimensional transfection systems

The strategy that combines the non-viral gene carrier and three-dimensional (3D) scaffold, based on reverse transfection methods, has been an attractive field for stem cell genetic manipulation since 1999. Jeffrey Bonadio et al. firstly reported the method of physically entrapping plasmid genes in a polymer matrix ('gene-activated matrix' or GAM). They managed to provide bone injury sites with sustained expression of plasmid DNA for more than 6 weeks by transplanting the GAM locally and demonstrated the induction of new bone in a stable, reproducible, dose-and time-dependent manner [79,80]. Since then, the definition of a tissue engineering matrix has changed dramatically from a material that functioned solely as an inert structural support for cell attachment, to serving as a more complex, dynamic environment for tissue development. In comparison with cells which are being cultured on 2D substrates, cells being cultured on 3D scaffolds adhere to the matrix through different sets of integrins and show a similar morphology as *in vivo*. Also, the signaling pathway activity and the following cellular response can obviously differ between the 2D and 3D environment. Furthermore, because of the presence of a large surface area from which to deliver DNA to cells, and to maintain an available pool of DNA for a long period without the aggregation lipoplex or polyplex, the transfection efficiency may be increased and sustained for a relatively long period [81]. It is believed that the matrix mechanics and fluid transport can influence many aspects of gene transfer. The signaling pathway activity – and ultimately the cellular response – can differ depending on whether the cells are present in 3D or 2D environment and also on the stiffness of the scaffold. Therefore, it was presumed that the difference in the mechanical properties of the 2D substrate and the 3D scaffold may account for the distinct gene expression of the cells cultured on them [80]. For example, the contraction of the collagen sponge with cells grown on it may lead to the collapse of the pore and thus hinder the nutrition and the transport of waste. In order to construct 3D scaffold, we used a non-woven PET fabric with excellent mechanical properties and a porous collagen with good biocompatibility. A sequent coating of anionic gelatin, pronectin and pullulan-spermine/pDNA complex (pDNA coding for luciferase or TGFβ-1 gene) was performed for the scaffolds. The BMSCs were then seeded to the scaffold for a three-dimensional reverse transfection. It was found that the transfection efficiency of the 3D systems reached its peak at around the 5th day. Generally, the gene expression level on a 2D substrate decreased rapidly but the cells cultured on the collagen sponge still retained a sustained gene expression on the 14th day and 21st day. Additionally, the cells in the PET fabric scaffold retained a high and sustained gene expression at all the observed time points within 3 weeks, which was supposed to be related to its biomimetic fibrous structure, when compared to the irregular collagen sponge [76,78]. In another example, the polyethylene glycol (PEG)-modified polyethylenimine (PEI) was incorporated into scaffolds by electrospinning and the target DNA was then adsorbed onto the electrospun nanofibers via the electrostatic interaction between the DNA and the PEI–PEG. This nanofiber-based gene delivery system exhibited a high transfection efficiency, in which >40% of the mesenchymal stem cells were transfected [82].

6. The application of genetically-manipulated stem cells and three-dimensional scaffolds, based biomimetic drug delivery systems in wound therapy

It is well known that the stem cells and/or gene therapy are becoming more and more important, not only in the basic research of medicine and biology but also in regenerative medical applications and current pharmaceutical design. With emerging knowledge and techniques of cellular- and molecular biology, it is believed that the utilization of the methodologies of cells transplantation and gene transfection into the development of advanced biomimetic drug

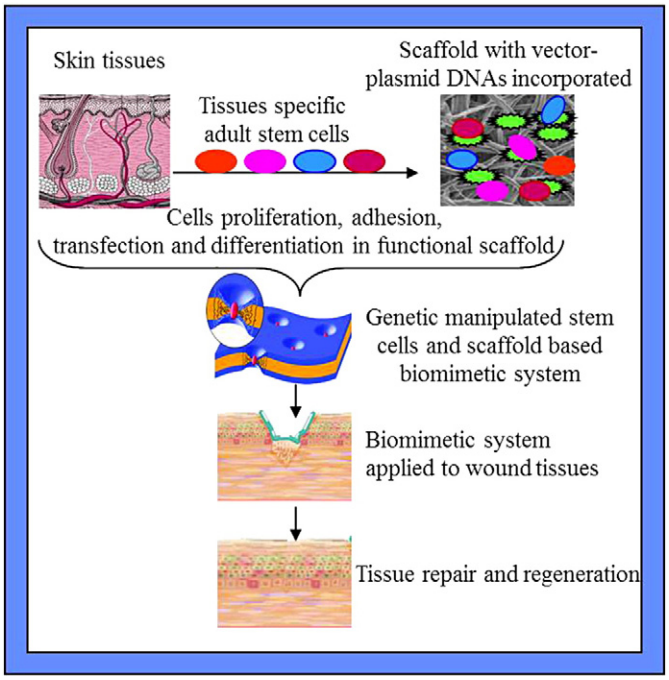

Fig. 3. Concise schematic diagram of the construct of biomimetic drug delivery system and its application *in vivo*: isolation and identification of tissue specific adult stem cells; ➔ co-culture of the stem cells with the functional scaffold (cell propagation, transfection and differentiation); ➔ the application of the biomimetic drug delivery system the injured tissue, as well as the degradation of the scaffold; ➔ the tissue repair and regeneration.

delivery system (BDDS) is necessary. In contrast to traditional methods, biomimetic drug delivery systems (BDDS) may possess one or more of the following features: a) Therapeutic cells, in most conditions, such as (genetic recombinant) adult stem cells with regenerative and differentiation characteristics, are used, in place of chemical agents or/and pure polypeptides, as therapeutic agents for the potential application of regenerative therapy, such as for the tissue repair and replacement. b) Besides functioning as therapeutic drugs, the adult stem cells also act as the vehicle for the transfer of plasmid DNA of growth factors and secreted the bioactive protein when applied to wound sites. c) Scaffolds made from biomimetic materials are constructed to mimic or even improve the characteristics of a natural extracellular matrix, emulate cell-extracellular matrix interactions and recapitulate the signaling cascades for providing an optimizing environment to facilitate cell recruiting, adhesion, proliferation, and the differentiation before *in vivo* application [83,84]. d) By encapsulating (for example) the bioactive proteins or non-viral plasmid DNAs within the scaffolds, the controlled release of growth factors/genes could be achieved over time, while retaining their bioactivity or efficiently transfecting the stem cells seeded in a sustained manner under physiological conditions [85]. In these advanced drug delivery systems with biomimetic features, the scaffold with high porosity acts both as the gene/protein release system for genetic cell manipulation, as well as being the carrier of the therapeutic cells or proteins, while (stem) cells may play roles both as the regenerative elements for tissue repair and the vehicle for the delivery of target genes to wound sites [86].

Fig. 3 shows a schematic diagram of the basic construct of the genetic stem cells and nano-scaffold-based biomimetic drug delivery system and its application in skin tissue repair. Recently, we have demonstrated the development of third-generation biomaterials to reduce steps in regenerating tissues, in which, it has been seen that hASCs and hBMSCs have similar bone-regenerating potential on gelatin scaffolds. It was shown that BMP-2, as an osteogenic differentiating stimulus, was sufficient for hASCs and hBMSCs, resulting in good bone formation. Combining third-generation smart biomaterials with integrated BMP-2 slow release and easily-accessible adipose-derived stem cells offers attractive new pathways for regenerating bone [87]. It was recently reported that linear polyethyleneimine was chemically conjugated to surface exposed amine groups of the nanofibrous matrix by a MMP-cleavable peptide linkage. DNA was then electrostatically incorporated on the matrix at various charge ratios of DNA to the immobilized LPEI. Animal experiments employing diabetic animals with dorsal ulcers revealed that the LPEI-immobilized nanofibrous matrix was superior to naked DNA or DNA-incorporated matrix without LPEI in terms of *in vivo* transfection efficiencies. Thus, controlled release of DNA in response to MMPs can be potentially applied to local gene delivery system for treating diabetic ulcers. The advantages of the BDDS over the traditional drug delivery systems lie in that: a) It delivers the therapeutic cells and genes with a biomimetic carrier/approach. b) These "living" BDDSs, which encode the genes into the stem cells and encapsulate the stem cells within the biomimetic matrix, are advantageous for controlled and continuous expression of genes and to improve the pharmacokinetics of easily degradable peptides and proteins by promoting the stem cells and genes against the immune cell- and antibody-mediated rejection and have the potential to produce an array of therapeutically-active substances. c) Biomimetic design of cell-loaded systems can improve cell performance as cells are immobilized in a functional scaffold that mimics the natural microenvironment of the cells and releases the target genes to be encoded. Table 1 shows several recently reported biomimetic systems which exhibited significantly therapeutic effects in tissue repair and regeneration.

7. Challenges and future perspectives

Cell transplantation, combined with gene modification, has augmented therapeutic efficacy dramatically due to its efficiency, specificity and safety. Although *ex vivo* cell culture is thought to be elaborate and costly, there is tremendous scope for using cell delivery therapeutics for repair of tissues and organs. In particular, the transfer of plasmid DNA of growth factors into stem cells – in order to boost or suppress expression of factors that are involved in tissue regeneration – is an intriguing therapeutic approach. As observed in various regeneration models, condensation of DNA with cationic polymers enhanced the duration of stem cell transfection when polyplexes were delivered from the 3D matrix *in vivo*. Thus, the use of stem cells as a biological basis for tissue engineering, coupled with acting as a vehicle for gene delivery, has created a wide range of possible new applications in gene therapy and tissue regeneration. Besides the major steps involved in this strategy, including: (i) synthesizing novel biodegradable or modified cationic polymers, which are less toxic to the cells and may exhibit a higher transfection efficiency on the adult stem cells (ii) constructing a 3D scaffold-based functional gene-activated matrix (iii) optimizing the scaffold transfection system and developing the reverse transduction method and (iv) *ex vivo* adhesion, genetic manipulation and differentiation of the stem cells in the gene-activated matrix, increasing research has been undertaken to identify more variables important to the efficacy of these approaches. For example, the mechanical stiffness of scaffold materials, to which cells adhere, has been shown to affect numerous aspects of cell behavior. Increasing stiffness led to an increase in polyplex uptake, decondensation and delivery to the cell nucleus, which was accompanied by an increase in cell proliferation. Accordingly, scaffold material properties, e.g. stiffness and surface morphology, may be manipulated to control transfection. In future, BDDS which respond to environmental changes such as pH, moisture or temperature – and that use the specific biochemistry of the injured site to control delivery of cells and genes – may represent an area deserving much more investigation. Furthermore, it can be expected that these BDDS may also be efficient in treating genetic diseases and their

Table 1
Recently reported biomimetic drug/gene delivery systems for tissue repair.

Formulation	Genes, proteins and/or cells	*In vitro* or *in vivo* models	Functions and applications	Refs.
Biomimetic chitosan–nanohydroxyapatite composite scaffolds	Pre-osteoblasts MC3T3-E1 cell line	*In vitro* MC 3T3-E1 culture	The biological responses of MC 3T3-E1 on nanocomposite scaffolds were superior in terms of improved cell attachment, higher proliferation, and well-spread morphology in relation to chitosan scaffold.	[88]
Poly-L-lysine coated polylactide scaffolds	TGF-β1 gene modified mesenchymal stem ells	Full-thickness articular cartilage defects in rabbits	Promote not only the ensuing differentiation of chondrocytes and the production of a cartilaginous matrix at the surface, but also the vigorous regeneration of subchondral bone.	[89]
Modified fibrin hydrogel matrice I	L1Ig6 incorporated	Chicken chorioallantoic membranes	The capillary network adjacent to the applied matrices appeared denser after stimulation with L1Ig6-modified matrices; than with native fibrin; Co-stimulation with L1Ig6 and VEGF-A165 or bFGF yielded a similarly dense appearance as stimulation with the individual growth factors alone.	[90]
Modified fibrin hydrogel matrice II	TG-peptide-DNA condensates containing the HIF-1αΔODD-plasmid DNA	Mouse skin wounds	50% more newly formed blood vessels were found and nearly 50% of these vessels were surrounded by smooth muscle cells indicating a higher degree of differentiation of these newly formed vascular structures than that of non transfected group.	[91]
Diffusion-controlled systems	HGF and FGF-2	Ischemic mouse hindlimb model	Enhanced blood vessel formation and produced more mature vasculature at lower doses than either factor alone.	[92]
Environmentally responsive systems	None	Injured rat cecum	Tissue adhesion between the PNIPAM–HA-treated cecum and adjacent tissues was significantly reduced as compared with that between non-treated tissue and adjacent tissues. The coating of a canine liver with PNIPAM–gelatin-containing PBS resulted in spontaneous gel formation on the tissues and subsequently suppressed bleeding.	[93]
Biologically inspired systems	three variant forms of vascular endothelial growth factor	*In vitro* angiogenesis of human umbilical vein endothelial cells	The factors contained fibrin matrix were competent in inducing endothelial cell proliferation, the matrix-bound forms being more effective than native VEGF, as well as competent in inducing endothelial progenitor cell maturation into endothelial cells.	[94]
Stimuli-responsive hydrogels incorporated with thermo-responsive polymers	Chondrocytes from adult rabbit scapular cartilage	*Ex vivo* culture and the cell phenotype were identified	Chondrocytes isolated from rabbit scapula can re-express chondrocyte phenotype in agarose culture and polymer gel culture but not in monolayer culture. Also, cultured chondrocytes can be easily recovered from polymer gel culture by simply lowering the temperature.	[95]

complications, e.g. diabetic skin ulcers, which require sustained delivery of growth factors to promote healing. However, as safety is always a key issue for any clinical application of cell therapy, a great deal of fundamental research on toxicological and immunological aspects, sterilization and upscaled production of the BDDS will be required and this will, in turn, present challenges which will necessarily form the basis of future clinical studies.

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